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#### **14. ABSTRACT**

KLF6 is a member of the Kruppel-like transcription factor family which was first identified as a tumor suppressor gene frequently inactivated in prostate cancer (PCa). A single germline SNP increases PCa risk and KLF6 gene alternative splicing to produce KLF6-SV1, which promotes tumor cell growth and metastasis. Since their original discoveries, many downstream cancer-relevant targets have been described as regulated by KLF6 and KLF6-SV1. However, less is known about the molecular pathways that regulate the function of these proteins. Therefore, the main purpose of this project was to identify the molecular pathways by which KLF6/KLF6-SV1 are involved in the initiation, progression and metastasis of PCa.

We have been highly successful in moving the project forward during the last two years. Indeed, our recent publication describes the work performed as part of Specific Aim #1 (Appendix). The paper describes our successful identification and characterization of the nucleo-cytoplasmic localization domains that regulate KLF6/KLF6-SV1 shuttling, protein stability and tumor suppressor function. The manuscript that acknowledges the support of this post-doctoral training award was published in PLoS ONE in 2009.

As a more translational outcome of this Aim, these results allowed us to establish a collaboration with a biopharmaceutical company to test nuclear export inhibitors as potential drugs for the treatment of prostate cancer. Work is now ongoing.

In addition to this, we were also requested to provide a research editorial on the KLF family and their role in human cancer, which was published soon after the start of this initial funding period. A copy of the manuscript, "The Krüppel traffic report: cooperative signals direct KLF8 nuclear transport", Rodríguez E, Martignetti JA. Cell Res. 2009 Sep;19 (9):1041-3, is included in the Appendix.

Beyond this, we have also started developing the constructs necessary for completion of the second part of Aim#1 and Aim#2. Specifically, we have generated and verified the mutants in the GSK3 $\beta$  and DNA damage PK domains in KLF6. We have also set up the conditions necessary for the DNA damage/cell cycle experiments with Doxorubicin.

As for the Aim#3, we have now demonstrated that both KLF6-SV1 and KLF6 bind to c-Myc, a known and critical oncogene highly relevant to prostate cancer development and outcome. Moreover, our preliminary results suggest that KLF6 overexpression may inhibit cell proliferation caused by c-Myc and transcriptional activation of c-Myc target genes both by qRT-PCR and luciferase promoter assays.

Taken together, these results begin to provide insight into how different cancer pathways regulate KLF6/KLF6-SV1 function, and in turn, how KLF6/KLF6-SV1 regulate these pathways. Ultimately, we believe these findings have provided insights into initiation, progression and spread of PCa and have opened opportunities for improving therapeutic strategies.

#### **15. SUBJECT TERMS**

KLF6, KLF6-SV1, NLS, NES, c-Myc, Half-life, Nucleo-cytoplasmic transport, tumor suppressor, prostate cancer development.

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## INTRODUCTION

KLF6 is a member of the Kruppel-like transcription factor family and it was first identified as a tumor suppressor gene frequently inactivated in Prostate cancer (PCa) (1, 2). A single germline SNP increases PCa risk and KLF6 gene alternative splicing to produce KLF6-SV1, which promotes tumor cell growth and metastasis (3, 4, 5). Since KLF6 was first discovered many downstream, cancer-relevant targets have since been described to be regulated by KLF6 and KLF6-SV1 (1, 6-11). However, less is known about the molecular pathways that regulate the function of these proteins. Therefore, the main purpose of this project was to identify the molecular pathways by which KLF6/KLF6-SV1 are involved in the initiation, progression and metastasis of PCa.

## BODY

**Training Accomplishments:** During these last two years, my mentoring and training as a future independent scientist have been achieved through different ways. Every week, I have met with my mentor, Dr. John A. Martignetti, to discuss and plan experiments, analyze data and define long-term studies. I have also presented my research project during our weekly lab meetings, in front of all fifteen members of our laboratory, which include an Assistant Professor, an Instructor, three other post-doctoral students, an MD/PhD student and research technicians.

Every year, I have presented my research findings in our Annual Departmental Research Seminar series, which is an institution-wide event where internationally-recognized researchers attend and present their work. In a similar way, I had also showed and discuss my research at the Work-in-Progress meeting that is held every week at the Department of Genetics and Genomic Sciences. In this forum, post-doctoral fellows have the opportunity not only to present their work and research plans but also to gain insight and criticism from all senior departmental clinical and research members.

I have attended one international meeting each year, including AACR in 2009 and IMPACT in 2011, where I had the opportunity to present my results and network with other scientist in the prostate cancer field.

Finally, and as extensions of my project and for future independent career studies, I have also initiated during these two years, a number of outside collaborations. The first one with Dr. Soichi Kojima, RIKEN Advanced Science Institute, Japan. This prostate cancer directed collaboration, is currently based on our studies exploring KLF6/KLF6-SV1 - c-Myc co-localization. For these studies, we are using FRET analysis techniques developed by Dr. Kojima's laboratory. The second collaboration is with Dr. Marc Glucksman, Professor, Biochemistry & Molecular Biology Director, Midwest Proteome Center, Rosalind Franklin University. Together, we have initiated a study examining the KLF6 prostate cancer proteome. The third collaboration is with Dr. Mark Chee (President, CEO, Prognosys Biosciences) and Dr. Waleed A. Hassen (Chief of Urology, Tawam Hospital, UAE/Johns Hopkins University) with whom we have initiated developing the framework for future studies to explore the genome sequence alterations associated with metastasis of prostate cancer. Finally, and most recently, we have established a collaboration with a biopharmaceutical company to test the use of nuclear export inhibitors in the treatment of prostate cancer cells.

As a follow up to this project, my mentor, Dr. Martignetti, and I have just applied for a DOD Idea development award within the PCRP in collaboration with Dr. Matthew Galsky, Assistant Professor of Urology and Medicine, Hematology and Medical Oncology (Mount Sinai School of Medicine), to study a set of RNA sequencing-selected genes as a new bone tropic signature that predicts PCa bone metastasis in human samples.

**Research Accomplishments:** During the past two years I have accomplished the majority of the experiments proposed in my original Statement of Work (SOW). The research has been highly successful as our first manuscript describing our findings was published in *PLoS ONE* in September 2010. A copy of the manuscript, which acknowledges the support of the CDMRP training grant, is provided in the Appendix to this report.

Moreover, and soon after the start of this initial funding period, we were also requested to provide a research editorial on the KLF family and their role in human cancer. A copy of the

manuscript, “The Krüppel traffic report: cooperative signals direct KLF8 nuclear transport”, Rodríguez E, Martignetti JA. Cell Res. 2009 Sep;19(9):1041-3, is included in the Appendix.

Below is a point-by-point description of the achievements of this grant in each one of the specific aims.

**Task 1. Define the role of nuclear shuttling signals and GSK3 beta in regulating KLF6/KLF6-SV1 subcellular localization.**

Subaim 1. We have completed the first part of this aim by identifying the protein domains that control KLF6/KLF6-SV1 subcellular localization. We have shown that these domains influence protein stability and KLF6 tumor suppressor function through the analysis of the expression of two well-known KLF6 target genes, p21 and E-cadherin. These subcellular localization domains include a Nuclear Localization Signal (NLS) located in the C-terminus region of KLF6, specifically in the first two zinc fingers that are part of the DNA binding domain; and a Nuclear Export Signal (NES) within the first 16 aa, in the N-terminus region. Mutants in the NLS have a longer half-life compared to wild type KLF6 and lose the ability to trans-activate p21 and E-cadherin target genes. Our results suggest that nuclear localization is a first step of regulation of KLF6 function.

In addition, mutants in the NES display a longer half-life consistent with the hypothesis, that an intact nucleo-cytoplasmic shuttling mechanism is necessary for proper protein degradation. Treatment with Leptomycin B proved that KLF6 is exported from the nucleus in a CRM1/Xpo1-dependent manner, and fusion of the NES to the Rev protein demonstrated that this domain has weak strength. This agrees with the role of KLF6 as a transcription factor. For a more detailed description of the experiments and results, we have provided our recently published manuscript, “Nucleo-Cytoplasmic Localization Domains Regulate Krüppel-Like Factor 6 (KLF6) Protein Stability and Tumor Suppressor Function”, as an attachment in the Appendix of this final report.

We also found, by co-immunoprecipitation experiments, that both KLF6 and KLF6-SV1 bind to the exportin CRM1/Xpo1. However, the NES mutant – predicted to the first 16 amino

acids - was found to be still bound (Figure 1). In this regard therefore, we could not prove whether or not the binding between KLF6/KLF6-SV1 and CRM1 was specific.

After publication of these results, a company biopharmaceutical company contacted us to establish collaboration. Together we are testing the use of a specific and novel class of nuclear export inhibitors for the treatment of PCa.

Subaim 2. For the second part of this aim, “role of GSK3 $\beta$  in KLF6/KLF6-SV1 subcellular localization”, we have already generated and validated the site-directed amino acid mutations that are part of the GSK3 $\beta$  domain found in KLF6/KLF6-SV1. Specifically, we have obtained the following mutants in the pGEX- KLF6 construct: S146A, S150A, S151A, S155A and the double mutant, S150A/S151A. The next step will be to perform *in vitro* phosphorylation assays to determine which amino acids are necessary for KLF6 phosphorylation by GSK3 $\beta$ . After finding the amino acids involved in KLF6 phosphorylation by this kinase, we will mutate the same amino acids in the pGFP-KLF6 construct to analyze the effect of these mutations in KLF6 subcellular localization.

### **Task 2. Studying a role for KLF6/KLF6-SV1 in the DNA damage response.**

We have generated and confirmed the KLF6 mutant in the putative DNA damage PK domain. We have used site-directed mutagenesis to simultaneously create the double mutant S59A/Q60A in the pGEX-KLF6 vector. Next step will be to perform *in vitro* phosphorylation experiments to probe that DNA damage PK phosphorylates KLF6 in the aforementioned amino acids.

We have also started setting up the conditions for the cell cycle analysis experiments. We have created a toxicity curve for Doxorubicin in BPH cells (Figure 2) from which we will choose the appropriate concentration of drug to treat BPH cells transfected with either KLF6 or the SQ mutant and analyze by FACS the cell cycle of the different cell lines.

### **Task 3. Studying the regulation of c-Myc oncogenic activity by KLF6/KLF6-SV1.**

We have demonstrated, using co-immunoprecipitation experiments, that not only KLF6-SV1 but also KLF6 binds c-Myc oncogene (Figure 3). In preliminary experiments, we demonstrated that over-expression of KLF6 can inhibit cell proliferation caused by c-Myc (Figure 4) and decrease transcriptional induction of several c-Myc target genes such as ATF3, Ki67 and Bcl-xl (Figure 5). These results could be showing a role of KLF6 in c-Myc's biology, which have been associated with PCa. To further investigate this, we have already created a c-Myc-RFP (Red Fluorescent Protein) construct in order to investigate KLF6 and c-Myc subcellular co-localization.

We have also performed luciferase promoter assays to analyze how protein-protein interactions between KLF6 and c-Myc could affect c-Myc transcriptional activity. We have used a CDK4 promoter (a known c-Myc target promoter) fused to the Luciferase reporter gene (kind gift from Dr. Fujii National Cancer Institute, Maryland) and co-transfected it together with c-Myc expressing plasmid and/or KLF6 expression plasmid in 293T cells. Despite we haven't been able to increase CDK4-luc expression after c-Myc overexpression, we have shown that the presence of KLF6 can decrease CDK4-luc activity when compared to c-Myc/pEGFP co-transfected cells. On the other hand, KLF6-SV1 seems to have a milder effect (Figure 6). We think that the problems encountered are due to the high levels of endogenous c-Myc present in the cell line used. We plan to repeat the experiments in a cell line with lower c-Myc expression.

The results obtained could indicate a potential role of KLF6 in inhibiting c-Myc transcriptional activity. More experiments are needed to fairly probe this hypothesis.

## **KEY RESEARCH ACCOMPLISHMENTS**

- Created EGFP fusion constructs and mutants of the KLF6 Nuclear Localization Signal (NLS) and Nuclear Export Signal (NES) to study the domains that control KLF6/KLF6-SV1 subcellular localization.
- Demonstrated that both an intact KLF6 NLS and NES are necessary for proper KLF6 protein degradation and that addition of the NLS to KLF6-SV1 reverts increased half-life to that of the wild type protein, which is shorter.

- Explored the role of the NLS in KLF6 function through two of its target, prostate cancer relevant genes, p21 and E-Cadherin. We used RT-PCR and Luciferase promoter assays to show that the NLS mutants cannot increase the transcription of these genes compared to the transcriptional induction after over-expression of the wild type protein.
- Demonstrated, using Leptomycin B treatment, that KLF6 nuclear export is CRM1-dependent and with the fusion to the Rev protein we had shown that KLF6 NES is of weak strength, which is in concordance with the role of this tumor suppressor as a transcription factor.
- Co-Immunoprecipitation experiments showed that KLF6 and KLF6-SV1 bind to the exportin CRM1/Xpo1. However, the NES mutant still bound to the exportin (Figure 1).
- Create mutants in the GSK3beta domain in pGEX-KLF6 construct.
- Create mutants in the DNA damage PK domain in pGEX-KLF6 construct.
- Toxicity curve for Doxorubicin in BPH cells (Figure 2).
- Shown that KLF6 can bind the oncoprotein c-Myc (Figure 3).
- Preliminary experiments have shown that KLF6 over-expression can inhibit c-Myc driven proliferation in PC3 cells and impair c-Myc target genes induction (Figures 4 and 5).
- Preliminary experiments have shown that KLF6 can inhibit CDK4 promoter induction, one well-known c-Myc targeted genes. More experiments are needed to know if this effect depends on c-Myc (Figure 6).

## **REPORTABLE OUTCOMES**

### Manuscripts:

- A manuscript has been recently published in PLoS ONE with the title “Nucleo-Cytoplasmic Localization Domains Regulate Krüppel-Like Factor 6 (KLF6) Protein Stability and Tumor Suppressor Function”. Rodríguez E, Aburjania N, Priedigkeit NM, DiFeo A, Martignetti JA. *PLoS One*. 2010. 5(9). pii: e12639.

- A research editorial on the KLF family and their role in human cancer was recently published with the title “The Krüppel traffic report: cooperative signals direct KLF8 nuclear transport”, Rodríguez E, Martignetti JA. Cell Res. 2009 Sep;19(9):1041-3. (See Appendices).

#### Abstracts:

Abstracts were presented for the annual Genetic and Genomics Sciences Department Retreat (MSSM) on December 2009 and 2010.

Another abstract was presented at the CDMRP IMPACT meeting 2011.

#### Presentations:

This work was presented at the Annual Genetic and Genomics Sciences Department Retreat (MSSM) both in 2009 and 2010 and as oral presentations in the Genetic and Genomics Sciences Department Work-in-Progress during the award period.

### **CONCLUSIONS**

We have been successful during the last two years in completing and moving forward with the research and training plans described in our original application. During this time, we have described the domains that control KLF6 nucleo-cytoplasmic shuttling and how these domains play a role in KLF6 protein half-life and tumor suppressor function. The manuscript describing these findings was published in PLoS ONE in 2009, where the support of the CDMRP Post-doctoral training award was acknowledged. Moreover we have started creating the tools necessary to study the role of GSK3 $\beta$  and DNA damage PK in KLF6/KLF6-SV1 subcellular localization and function. We have moved forward in studying the relationship between KLF6/KLF6-SV1 and the oncogene c-Myc, which is frequently over-expressed in PCa (12). Taken together, these results have brought more insights in the role of KLF6 and KLF6-SV1 in prostate cancer biology. We have begun to understand how different pathways regulate these proteins' function and here we have opened the door for the use of new therapeutic drugs, such as nuclear export inhibitors, in the treatment of PCa.

Moreover, a research editorial on the KLF family and their role in human cancer was also published soon after the start of this initial funding period (See Appendix).

Finally, I want to thank the funding institution for giving me the opportunity to develop both the training and research plans as defined during this award period, as they were critical to improve the PI's knowledge by providing her academic and scientific training and the ability to identify additional collaborators necessary for future work as an independent investigator in the areas of PCa biology and treatment.

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APPENDIX

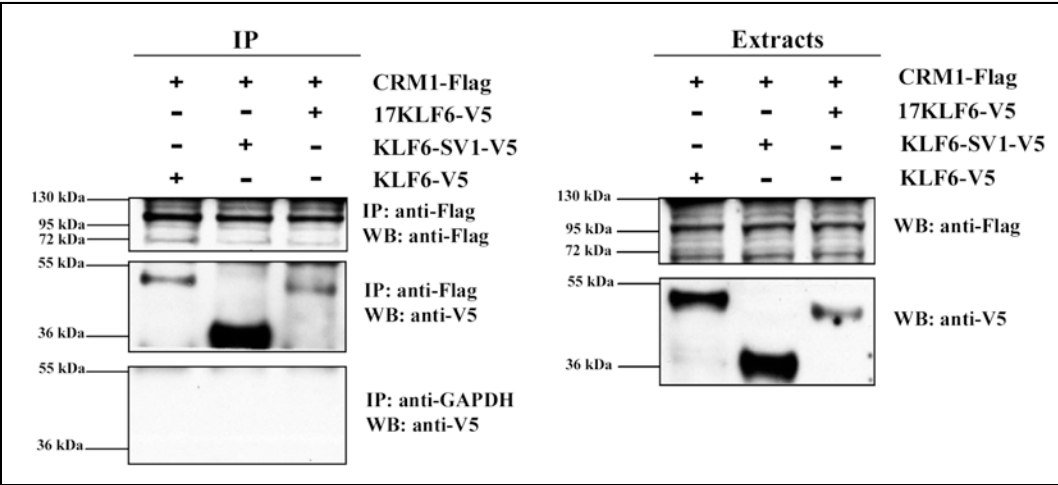


Figure 1. KLF6/KLF6-SV1 and the NES mutant (17KLF6) bind to CRM1. Co-immunoprecipitation between KLF6, KLF6-SV1 or 17KLF6 and CRM1 exportin in 293T cells.

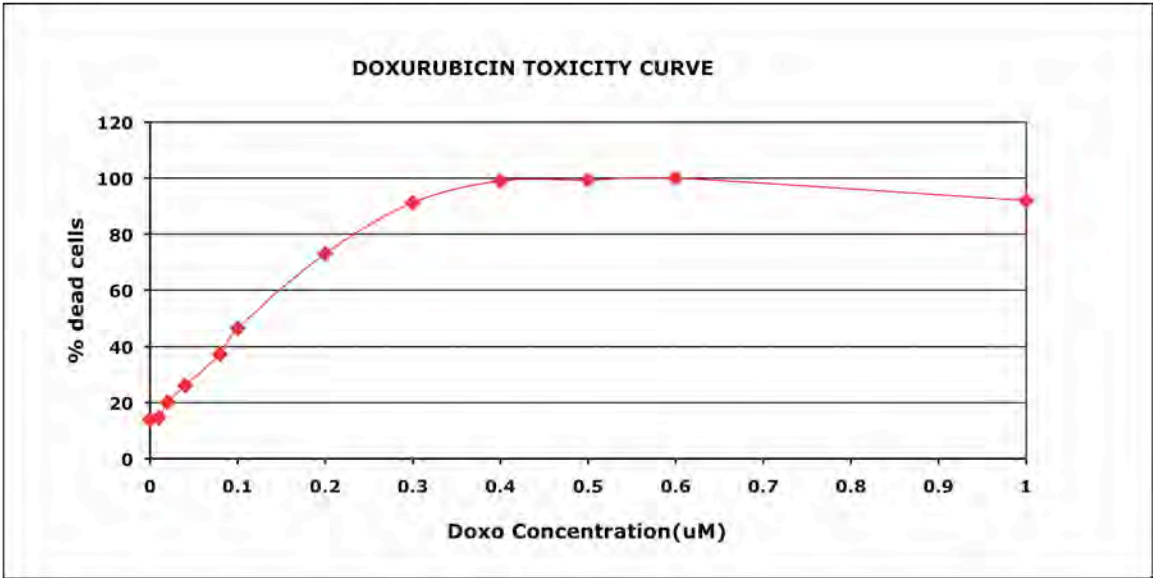
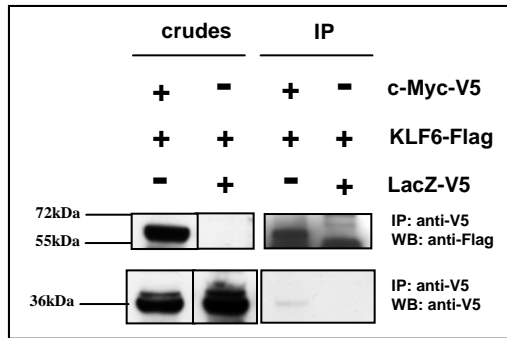
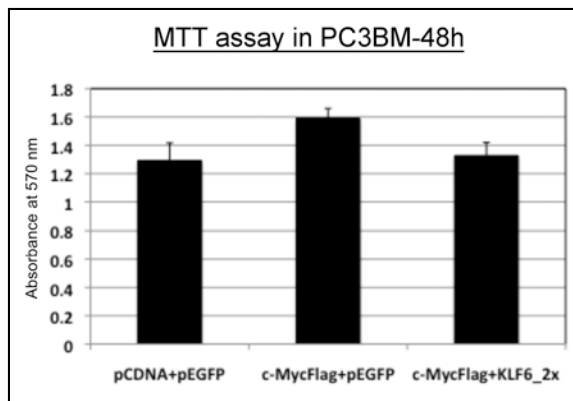


Figure 2. Doxorubicin Toxicity Curve in BPH cells. Different concentrations of doxorubicin were used to treat BPH cells for 48h. Cell viability was measured by cell counting. Percentage of cell death is represented.



**Figure 3.** KLF6 binds c-Myc. Co-immunoprecipitation between KLF6 and c-Myc in 293T cells. Cells were transfected with both KLF6-Flag and c-Myc-V5 or LacZV5 control vector.



**Figure 4.** KLF6 over-expression could be inhibiting c-Myc proliferation in PC3BM cells. PC3BM cells were transfected with empty vectors, c-Myc or c-Myc plus twice the same amount of KLF6. Next day, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent was added and absorbance was measured at 570 nm.

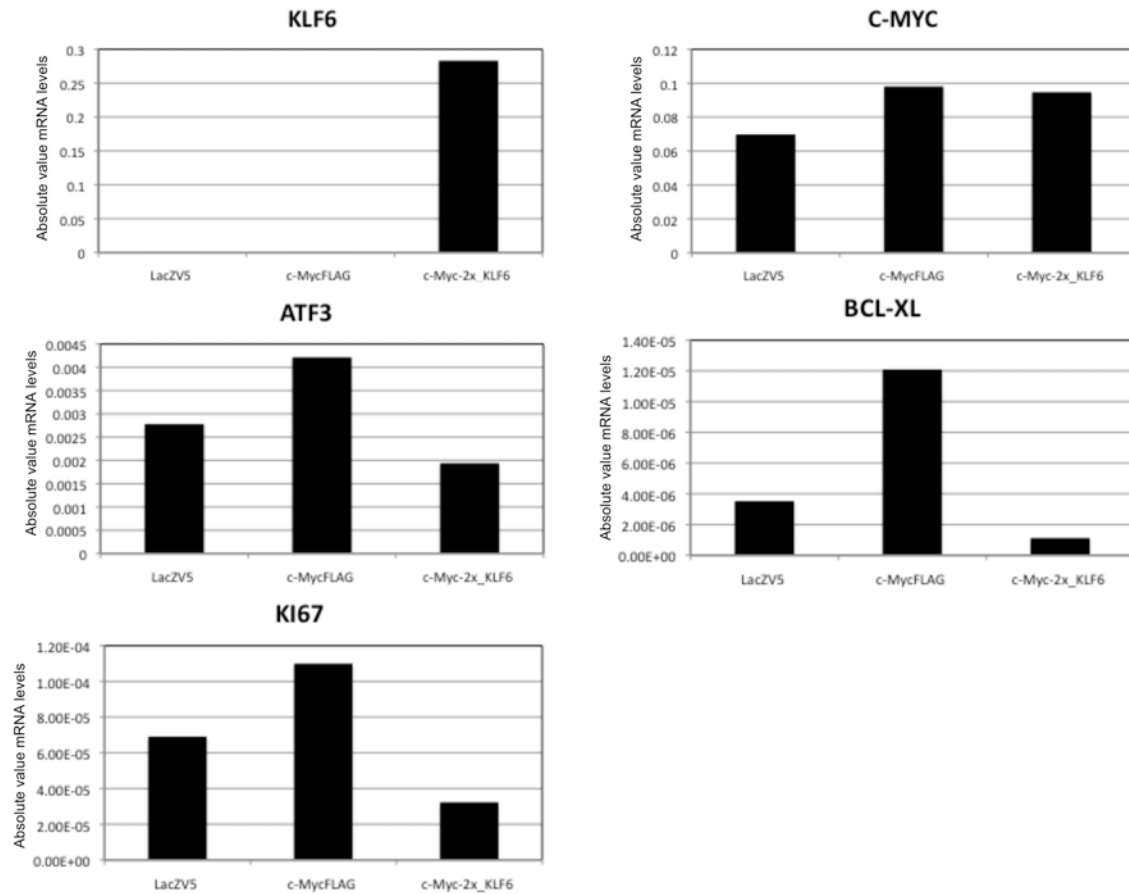


Figure 3. KLF6 over-expression could be inhibiting c-Myc induction of target genes. 293T cells were transfected with empty vector, c-Myc or c-Myc and twice the same amount of KLF6. Cells were harvest for RNA extraction, cDNA was generated by reverse transcription using random primers (Promega). An ABI PRISM 7900HT Sequence Detection System (Applied

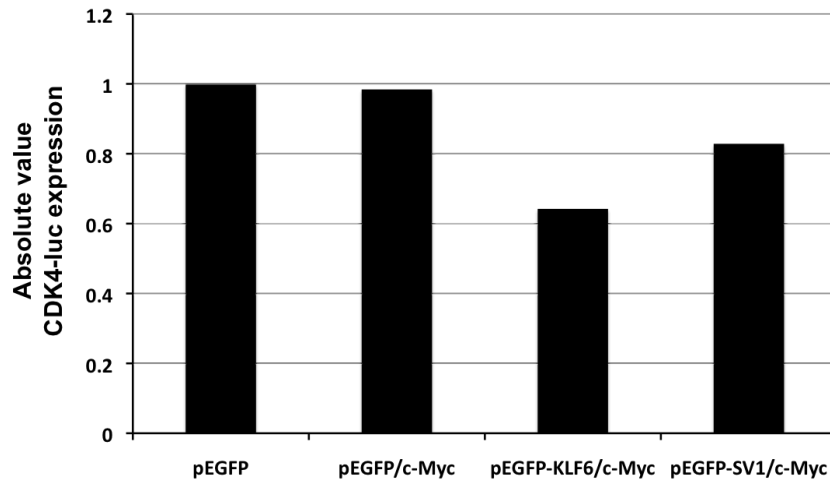


Figure 6. CDK4-luciferase promoter assay in 293T cells to study the effects of KLF6/KLF6-SV1 in c-Myc transcriptional activity. Cells transfected with the different constructs were harvested after 24 h. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega), following the manufacture protocol. Luciferase units for each sample were normalized by the value for the pEGFP control transfected cells.

# Nucleo-Cytoplasmic Localization Domains Regulate Krüppel-Like Factor 6 (KLF6) Protein Stability and Tumor Suppressor Function

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## Abstract

**Background:** The tumor suppressor KLF6 and its oncogenic cytoplasmic splice variant KLF6-SV1 represent a paradigm in cancer biology in that their antagonistic cancer functions are encoded within the same gene. As a consequence of splicing, KLF6-SV1 loses both the C-terminus C<sub>2</sub>H<sub>2</sub> three zinc finger (ZF) domain, which characterizes all KLF proteins, as well as the adjacent 5' basic region (5BR), a putative nuclear localization signal (NLS). It has been hypothesized that this NLS is a functional domain critical to direct the distinct subcellular localization of the tumor suppressor and its splice variant.

**Methodology/Principal Findings:** In this study, we demonstrate using EGFP fusion constructs that KLF6/KLF6-SV1 nucleo-cytoplasmic transport is not regulated by the 5' basic region but activated by a novel NLS encoded within the ZF domain, and a nuclear export signal (NES) located in the first 16 amino acids of the shared N-terminus sequence. We demonstrate KLF6 nuclear export to be Crm1-dependent. The dysregulation of nucleo-cytoplasmic transport when disrupting the KLF6 NLS using site-directed mutagenesis showed that its integrity is necessary for appropriate protein stability. Moreover, these mutations impaired transcriptional induction of two KLF6 well-characterized target genes, E-cadherin and p21, as shown by RT-PCR and luciferase promoter assays. The addition of the ZF domain to KLF6-SV1 results in its nuclear localization and a markedly decreased half-life similar to wild type KLF6.

**Conclusions/Significance:** We describe the domains that control KLF6 nucleo-cytoplasmic shuttling and how these domains play a role in KLF6 protein half-life and tumor suppressor function. The results begin to mechanistically explain, at least in part, the opposing functions of KLF6 and KLF6-SV1 in cancer.

**Citation:** Rodríguez E, Aburjania N, Priedigkeit NM, DiFeo A, Martignetti JA (2010) Nucleo-Cytoplasmic Localization Domains Regulate Krüppel-Like Factor 6 (KLF6) Protein Stability and Tumor Suppressor Function. PLoS ONE 5(9): e12639. doi:10.1371/journal.pone.0012639

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## Introduction

KLF6 is a tumor suppressor gene and member of the Krüppel-like factor family of transcriptional regulators involved in development and differentiation as well as in growth signaling pathways, apoptosis, proliferation and angiogenesis [1,2]. The tumor suppressor function of KLF6 has been widely confirmed through its loss and mutation in a number of cancers and the ability to reduce colony formation in cultured cells [1,3,4–14]. Like all members of the KLF family, KLF6 is characterized by three C-terminus C<sub>2</sub>H<sub>2</sub> zinc fingers (ZF) that form the DNA binding domain and an N-terminus activation domain [15].

Intriguingly, KLF6 is alternatively spliced into KLF6-SV1, a cytoplasmic protein that lacks the canonical KLF family DNA binding domain and the contiguous 5' basic region (5BR), considered a putative NLS, which are both replaced by a novel C-terminal 21 amino acids (16, Figure 1A). While KLF6-SV1 appears to localize exclusively in the cytoplasm, KLF6 is present in both the nucleus and cytoplasm [16]. To date, the distinct subcellular localization differences between KLF6 and KLF6-SV1

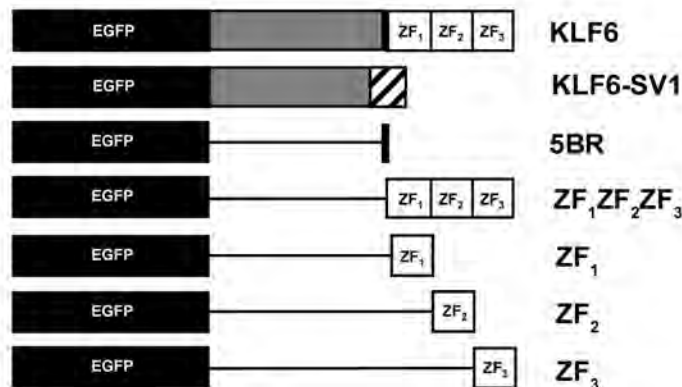
have been attributed, respectively, to the presence or absence of the 5' basic region. KLF6-SV1 was first shown to promote tumor growth, cancer development and metastasis in prostate cancer (PCa) [1]. Since its original identification in PCa, increased expression of this C-terminus truncated splice variant has been correlated with metastasis and poor survival not only in prostate cancer [1,16,17] but also in nasopharyngeal carcinoma [14], colorectal cancer [6], lung cancer [18], hepatocellular carcinoma [8], glioblastoma [4], ovarian cancer [3], head and neck squamous cell carcinoma [13] and pancreatic cancer [19]. Given the cancer-relevant and antagonistic functions of KLF6 and KLF6-SV1 it will be important to define the functionality of the putative NLS, the 5BR, as well as the role of nucleo-cytoplasmic shuttling in regulating KLF6/KLF6-SV1 function.

Regarding subcellular localization domains, the putative NLS has been shown to be functional in KLF1 and KLF4. Moreover, the KLF zinc finger domain has also been implicated in driving nuclear localization of these proteins [20–23]. On the other hand, only KLF5 has been demonstrated to possess a nuclear export signal (NES) [24]. In general, subcellular trafficking depends on

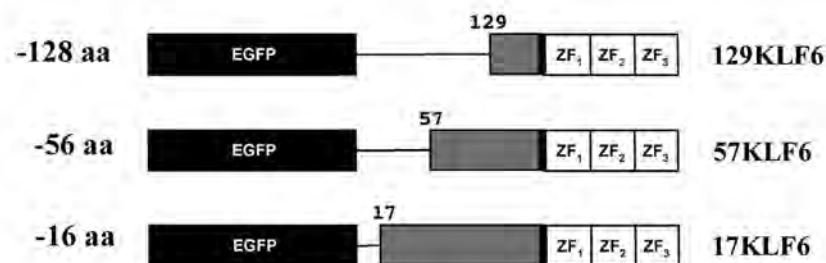
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**Figure 1. Diagram of the different EGFP constructs.** A, KLF6 and KLF6-SV1 protein sequences. All Leu (L) and Ile (I) residues are highlighted in red. The 16 amino acids (aa) that form the KLF6 NES are in bold. Other hydrophobic aa within the NES are underlined. The continuous underlines in the C-terminus of the KLF6 sequence represent the three zinc fingers. The aa which differ between the two proteins are highlighted in blue. B, Diagram of the EGFP constructs used to interrogate and define the KLF6 NLS. C, Diagram of the N-terminus deletions used to identify and investigate the KLF6 NES. doi:10.1371/journal.pone.0012639.g001

the presence of specific functional domains within protein sequences. Nuclear localization signals (NLS), whether classical (monopartite or bipartite) or not, are motifs that direct proteins

into the nucleus [25–28]. These signals, which are recognized by protein carriers called importins, are characterized by the presence of basic residues, Lys and Arg. In many cases these signals are

located near or within other important domains that regulate protein activity [29]. For example, in many transcription factors, NLSs are localized in the proximity of their DNA binding domains [20,30]. On the other hand, nuclear export signals (NES), which are recognized by exportins and are characterized by hydrophobic amino acids [31,32], are responsible for the transport of proteins out of the nucleus, back to the cytoplasm. The most common protein involved in exporting cargo from the nucleus is the transporter protein Crm1/Xpo1, first discovered in yeast [33–36].

Subcellular localization and protein turnover are two related events that are tightly regulated and control the function of different tumor suppressor proteins. Examples include Rb [37], PTEN [38], BRCA1, p53 and FOXO [39,40]. Mutations in the corresponding nuclear import-export domains of these proteins disrupt transporter binding, which, in turn, alter their nucleo-cytoplasmic shuttling and, therefore, their normal spatiotemporal dynamics. Among different consequences, protein mislocalization results in abnormal protein turnover and altered function that can promote cell transformation and tumor development [39,41–42].

In this work, we demonstrate that the functional KLF6 NLS is contained within the zinc finger domain but does not include the highly conserved contiguous 5′ basic region (5BR). Moreover, we also identify and characterize a functional NES that regulates KLF6 nucleo-cytoplasmic shuttling in a Crm1-dependent manner. Together, these domains appear to regulate KLF6 nucleo-cytoplasmic transport as well as regulate the half-life of both KLF6 and KLF6-SV1. In sum, these results begin to explain the differences in subcellular localization, half-life and, possibly, function between KLF6 and KLF6-SV1 and how KLF6 gene mutations in these domains and the increase in alternative splicing may result in tumorigenesis.

## Results

### The KLF6 C-terminus zinc finger domain defines nuclear localization

To investigate whether KLF6 possesses a functional NLS, we generated a series of four constructs encoding truncated KLF6-derived proteins fused to the reporter protein EGFP. The fusion proteins consisted of: pEGFP-KLF6, which encodes the full length KLF6 protein; pEGFP-SV1, full length KLF6-SV1; pEGFP-5BR, the 5′ basic region (5BR); and pEGFP-ZF<sub>1</sub>ZF<sub>2</sub>ZF<sub>3</sub>, the entire KLF6 zinc finger (ZF) domain (Figure 1B). The constructs were transfected into HeLa cells and 293T cells and after 24 h their subcellular localization was analyzed by fluorescence microscopy. In agreement with our previous immunohistochemistry findings [16], KLF6 was present both in the nucleus and cytoplasm with areas of intense perinuclear staining, while KLF6-SV1 localized exclusively in the cytoplasm (Figure 2 and Figure S1). The 5′ basic region alone was unable to drive EGFP into the nucleus and cells showed an equal nucleo-cytoplasmic distribution, similar to EGFP control cells. In contrast, cells over-expressing the complete ZF domain had an exclusive nuclear localization pattern (Figure 2 and Figure S1).

To dissect the relative importance of each one of the three ZFs with regard to KLF6 nuclear localization, we engineered three additional constructs wherein each ZF was independently expressed and interrogated: pEGFP-ZF<sub>1</sub>, pEGFP-ZF<sub>2</sub> and pEGFP-ZF<sub>3</sub> (Figure 1B). After expressing these constructs in HeLa cells, ZF<sub>1</sub> localization was shown to be similar to that of wild type KLF6 (Figure 2). Namely, ZF<sub>1</sub> had predominant cytoplasmic staining with some nuclear and perinuclear expression. In contrast, ZF<sub>2</sub> and ZF<sub>3</sub> resulted in a more equivalent nucleo-cytoplasmic distribution. Nonetheless, ZF<sub>2</sub> expressing cells had a

more easily distinguishable nuclear staining pattern than ZF<sub>3</sub> cells (Figure 2).

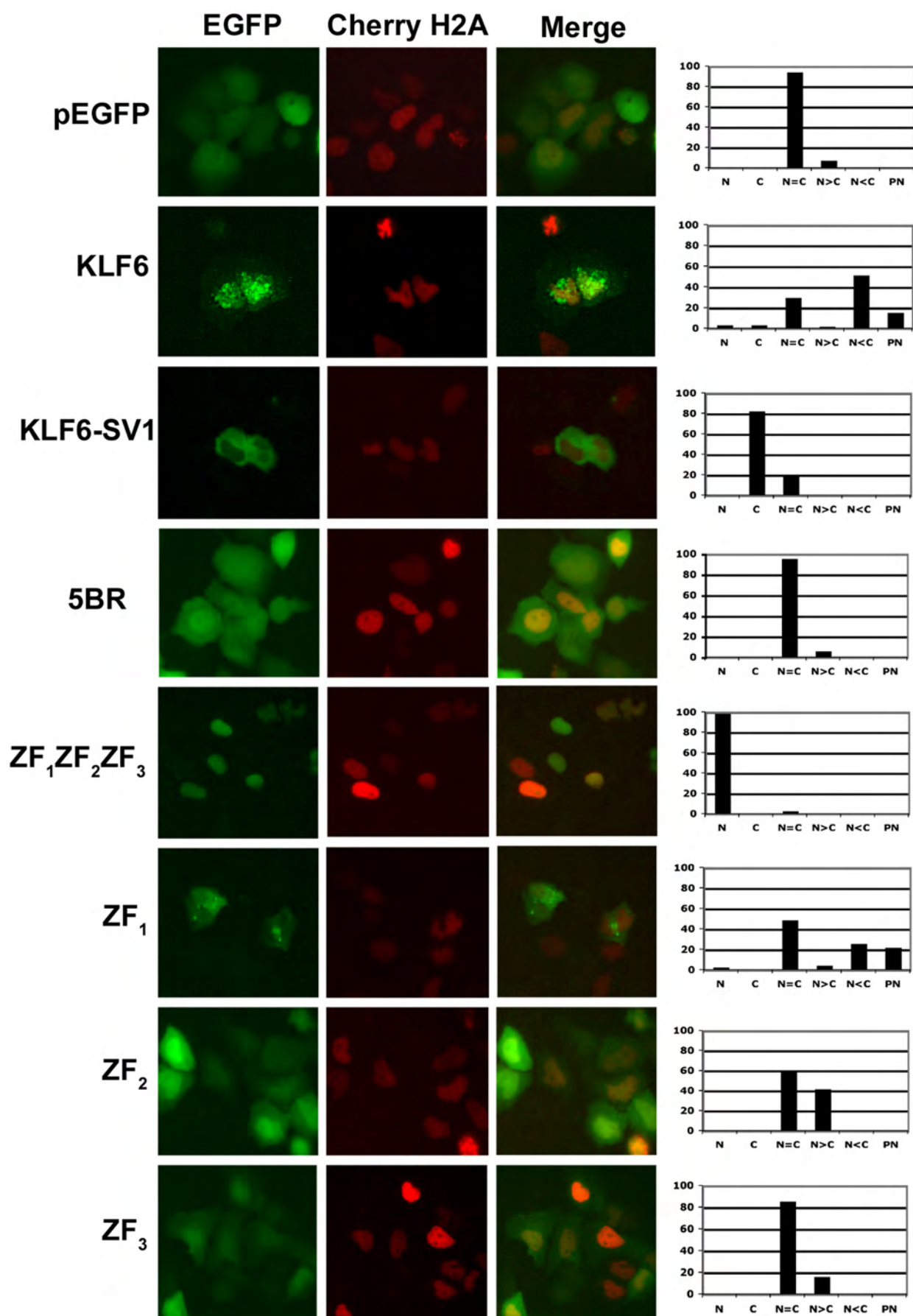
It has been previously proposed that the basic residues within the ZFs of Krüppel-like factor KLF1 may represent a common NLS motif for all KLF members [21]. It has also been demonstrated that mutations in these basic residues only affect transport but not DNA binding of KLF1 [21]. Given these previous findings, we therefore mutated the basic residues within ZF<sub>1</sub> and ZF<sub>2</sub> to more precisely define and map the amino acids involved in KLF6 nuclear localization. Using site-directed mutagenesis we replaced a number of Arg and Lys residues within the ZF domain with Ala (Figure 3A). In total, 11 residues were replaced in both zinc finger domains. The loss of the 5 basic residues in ZF<sub>1</sub> drastically decreased the number of cells with nuclear and perinuclear localization and increased the number of exclusively cytoplasmically staining cells. Replacement of the 6 basic residues in ZF<sub>2</sub>, along with the altered ZF<sub>1</sub>, further increased the number of cells with cytoplasmic KLF6 localization (Figure 3B). This suggested that in our experimental system, while ZF<sub>1</sub> may be the main driver of KLF6 nuclear localization, ZF<sub>2</sub> plays a minor but important role.

Given these findings, one hypothesis that would explain the cytoplasmic localization of KLF6-SV1 is that absence of the ZF domain, and not the 5′ basic region, results in its distinct subcellular localization. To test this, we engineered a KLF6-SV1 construct that possessed all three KLF6 ZFs (KLF6-SV1-Z<sub>1</sub>Z<sub>2</sub>Z<sub>3</sub>). The addition of the complete ZF domain to the chimeric protein resulted in complete re-localization of KLF6-SV1 from the cytoplasm into the nucleus (Figure 3B).

### KLF6 has an NES that is Crm1-dependent

The nucleo-cytoplasmic localization of KLF6 together with the presence of a functional NLS supported the idea that KLF6 could also harbor a functional nuclear export signal (NES). As a first approach to identify this NES, and in order to investigate whether KLF6 nuclear export was mediated by Crm1, we treated HeLa cells expressing EGFP-tagged KLF6, KLF6-SV1 or EGFP with the Crm1 inhibitor Leptomycin B (LMB) [43,44]. In stark contrast to non-LMB treated cells that continued to display both nuclear and cytoplasmic staining of KLF6, LMB treatment resulted in marked KLF6 nuclear accumulation (Figure 4A). Surprisingly, LMB treatment of KLF6-SV1 transfected cells also resulted in nuclear enrichment (Figure 4A). This finding suggests that KLF6-SV1, like the wild type protein KLF6, can also translocate to and exist within the nucleus. We did not explore further whether this was the result of an additional NLS within the primary sequence or possibly through shuttling (“piggy-backing”) with another protein, possibly endogenous KLF6.

For mapping the KLF6 NES domain, we initially used an NES prediction program (<http://www.cbs.dtu.dk/services/NetNES/>). Using this *in silico* approach only one amino acid with a high score for a putative NES was identified, Ile15. However, manual inspection of the sequence revealed a large number of hydrophobic residues, a common feature of NESs [32], within the first 132 amino acids of the KLF6 protein sequence (highlighted in red in Figure 1A). In order to test whether this region contained a functional NES within this region, we generated three overlapping N-terminus serial deletions and tested their ability to direct transport (Figure 1C). Before microscope visualization, we expressed and analyzed by Western-blot all three truncated proteins (17KLF6, 57KLF6 and 129KLF6), confirming that they were stable and expressed in similar amounts (Data not shown). As displayed in Figure 4B, all three constructs, lacking the first 128 aa, 56 aa and 16 aa, respectively, remained in the nucleus suggesting



**Figure 2. The KLF6 functional NLS resides within the zinc finger domain.** Co-localization of KLF6, KLF6-SV1, 5BR or the ZFs EGFP constructs together with Cherry-H2A, which was used to show nuclear staining. Localization of the different constructs was observed by fluorescence microscopy. Graphs with the percentage of cells with the different localization are shown on the right. N, Nuclear localization, C, Cytoplasmic localization, N=C, Nuclear and cytoplasmic distribution within the same cell is equal, N>C, Nuclear localization is more intense than cytoplasmic localization, N<C, Nuclear localization is less intense than cytoplasmic localization, and PN, perinuclear localization.  
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that all of them lacked a functional NES. Thus, based on the shortest deletion, 16aa, at least one functional NES must exist within this domain and exist within this region.

We then sequentially mutated each of the 9 hydrophobic residues within these first 16 aa to Ala using site-directed mutagenesis (Figure S2). The effect of these mutations on nuclear localization was easily discernible. Only mutations in Val3, Met6, Phe10, Leu13 or Ile15 increased KLF6 nuclear sequestration (Figure S2).

The nuclear export rate of a protein depends on the activity of its NES, which in turn is determined by the strength and accessibility to the domain [45]. To gain an approximate understanding of this, we measured the relative strength of the KLF6 NES. Using the system first described by Henderson *et al.*, 2000 we compared the KLF6 NES to that of the human immunodeficiency virus type I (HIV-I) Rev protein. We used three different constructs: pRev1.4-EGFP, encoding an NES-deficient Rev protein; pRev1.4 (NES3)-EGFP, expressing Rev plus its own NES; and pRev1.4 (KLF6 NES)-EGFP that replaces the Rev NES with that from KLF6. HeLa cells over-expressing the NES-deficient Rev protein (pRev1.4-EGFP) showed complete nuclear localization whereas the Rev NES containing protein (pRev1.4 (NES3)-EGFP) was exclusively cytoplasmic (Figure 5). However, replacement of the Rev NES with the 16 aa KLF6 NES resulted in partial cytoplasmic relocation of Rev. Treatment of all 3 transfected cell lines with LMB resulted in complete relocation of EGFP into the nucleus thus suggesting again the Crm1-dependent nature of the KLF6 NES.

### Nucleo-cytoplasmic transport regulates KLF6 and KLF6-SV1 protein stability

When analyzing their subcellular localization, we noted that cells over-expressing KLF6 showed, in general, less fluorescence compared to those over-expressing KLF6-SV1 or EGFP. Moreover, the different chimeric and mutated KLF6 proteins revealed that fluorescence intensity varied between constructs but not between experiments (data not shown). As protein stability has been linked to protein subcellular localization we investigated whether the half-life of the NLS and NES mutants was different. We treated HeLa cells over-expressing the different proteins with cycloheximide (CHX) to inhibit *de novo* protein synthesis and then harvested protein extracts at different time points for Western-blotting. In accord with previous findings [46], wild type KLF6 half-life was ~18 min (Figure 6).

As predicted, changes in the NLS and NES sequences affected protein stability. The ZF<sub>1</sub> mutant (KLF6-Z<sub>1</sub>A<sub>5</sub>), doubled KLF6 half-life to ~40 min. Additional mutations in ZF<sub>2</sub> (KLF6-Z<sub>1</sub>A<sub>5</sub>Z<sub>2</sub>A<sub>6</sub>) further significantly increased the half-life (Figure 6). Deletion of the NES (17KLF6) also resulted in a markedly increased half-life compared to the wild type protein. The half-life of 17KLF6 was longer than 1 h. Point mutations in one of the mapped critical amino acids (mutant L13AKLF6) were also sufficient to increase KLF6 half-life, having the same effect on half-life as deletion of the complete NES (Data not shown).

KLF6-SV1 has a markedly longer half-life compared to KLF6, >1 h [47]. To determine if KLF6-SV1 stability was also influenced by nucleo-cytoplasmic transport, we added the KLF6

NLS to KLF6-SV1 with the aim of restoring nuclear localization. We generated the chimeric protein SV1-Z<sub>1</sub>Z<sub>2</sub>Z<sub>3</sub>. After transfection in HeLa cells, SV1-Z<sub>1</sub>Z<sub>2</sub>Z<sub>3</sub> restored not only KLF6-SV1 nuclear localization but also resulted in a shorter half-life, ~19 min, similar to the half-life of the wild type protein KLF6 (Figure 6).

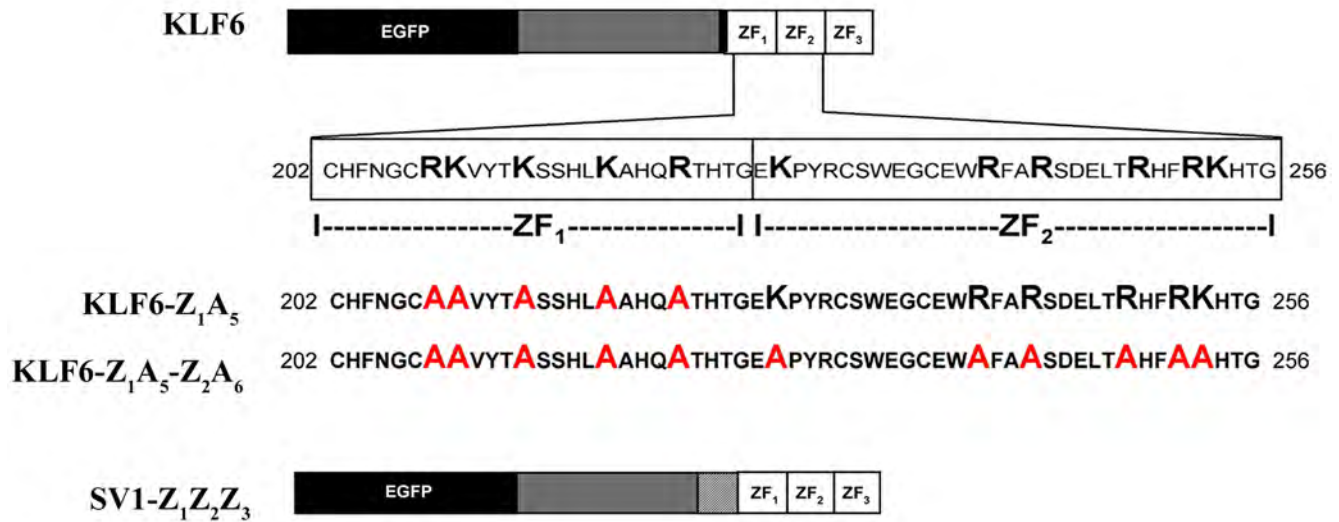
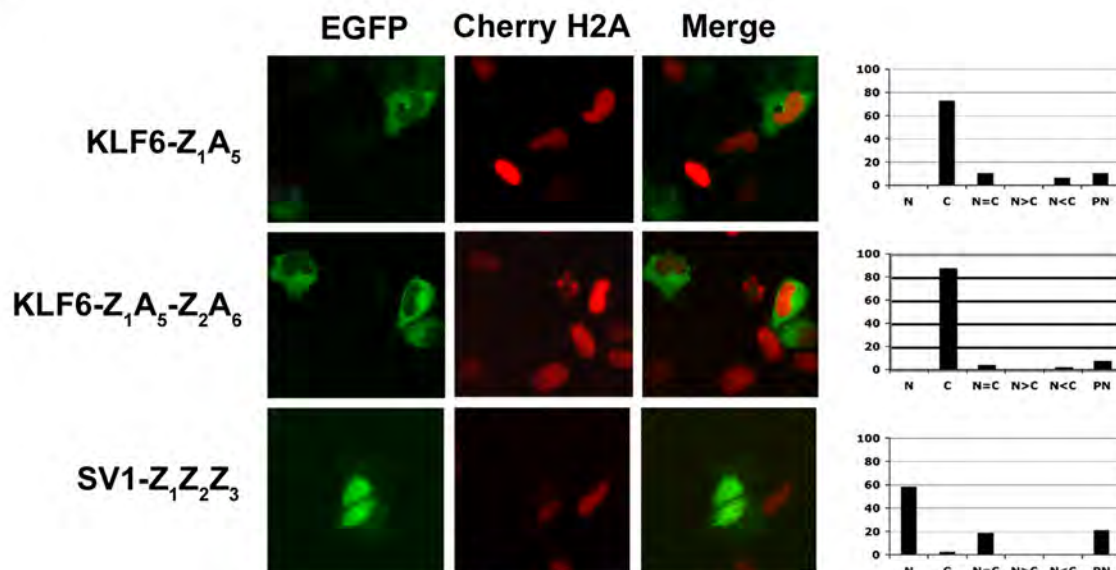
### Nuclear localization affects KLF6 tumor suppressor function

We next investigated whether the differing subcellular localizations of KLF6 and KLF6-SV1 may in part underlie their antagonistic functions. We chose two well-characterized KLF6 transcriptional targets, the transmembrane protein E-cadherin and the cyclin-dependent kinase inhibitor p21. The expression of these two genes has been shown to be increased by wild type KLF6 but not KLF6-SV1 [1,17,48]. We used HeLa cells over-expressing KLF6, KLF6-SV1 or two NLS mutants (KLF6-Z<sub>1</sub>A<sub>5</sub>, KLF6-Z<sub>1</sub>A<sub>5</sub>Z<sub>2</sub>A<sub>6</sub>) to measure the levels of expression of E-cadherin, by RT-PCR, and p21, by both RT-PCR and luciferase promoter assays. As shown in Figure 7A, cells over-expressing wild type KLF6 doubled E-cadherin expression compared with control vector ( $p<0.005$ ). Over-expression of KLF6-SV1 or either of the KLF6 NLS mutants had no effect on E-cadherin expression (Figure 7A). Similarly, cells over-expressing KLF6 increased endogenous p21 expression ~20% ( $p<0.05$ ) (Figure 7B), and about 4-fold increase when a p21 promoter fused to luciferase gene was used ( $p<0.005$ ) (Figure S3). No changes were detected in the levels of p21 in cells over-expressing KLF6-SV1 or the two KLF6 NLS mutants in neither one of the experiments. Figure 7C shows the level of expression of the different constructs transfected.

### Discussion

In this work we define and characterize a number of novel regulatory domains and test the mechanisms involved in nucleo-cytoplasmic transport of KLF6 and KLF6-SV1. In turn, these domains seem to be necessary for regulating protein turnover and help to establish functional differences between KLF6 and KLF6-SV1, which have both been shown to play important roles in cancer initiation, progression and survival and for predicting outcome. For example, addition of the KLF6 NLS to KLF6-SV1 results in nuclear localization of this oncogenic protein while markedly decreasing its half-life. Conversely, removing the native NLS sequence from KLF6 resulted in its loss of nuclear targeting but also its inability to activate E-cadherin and p21 transcription.

KLF6 is frequently inactivated in a number of human cancers. Inactivation can occur through multiple mechanisms including mutation, loss of heterozygosity (LOH), promoter hypermethylation and/or an increase in alternative splicing [1,5–7,14,16,46,49]. Examination of the published KLF6 mutations demonstrates that indeed a number of the cancer-defined mutations occur in the NLS and NES domains (Figure 8). Three mutations map into the NLS: S215F has been identified in astrocytoma, glioblastoma and meningioma [46], R243K in nasopharyngeal carcinoma [14], and L217S in prostate cancer [1,5]. In the NES domain, two mutations, D2G and M6V, have also been identified in astrocytoma, glioblastoma and meningioma [50]. Of note, in this

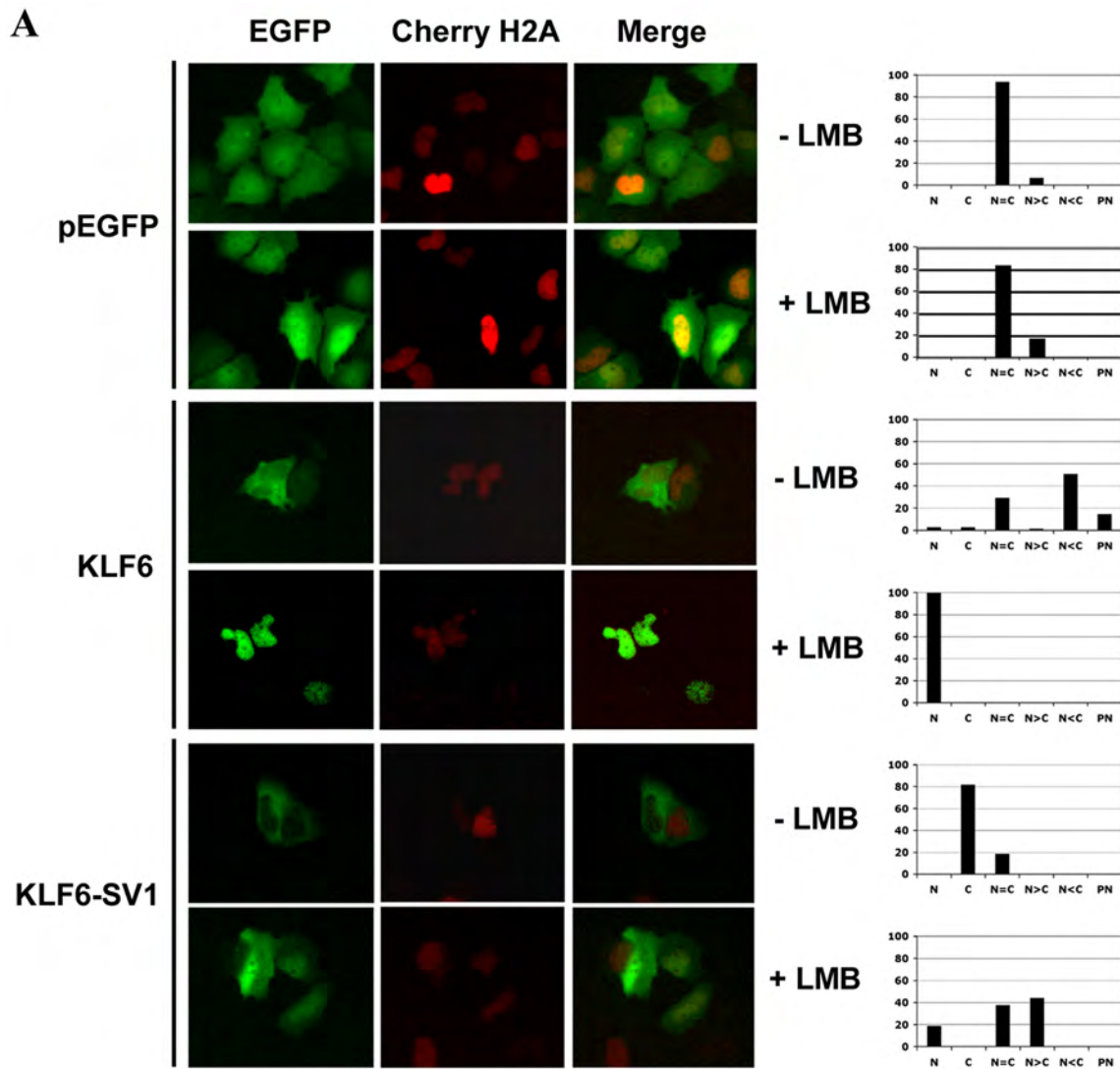
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**Figure 3. Mutations within the ZFs affect KLF6 nuclear transport.** A, Cartoon showing the Ala replacement mutations introduced in ZF<sub>1</sub> and ZF<sub>2</sub> and the structure of the chimera SV1-Z<sub>1</sub>Z<sub>2</sub>Z<sub>3</sub>. B, Subcellular localization of the constructs following transfection in Hela cells. Cherry-H2A construct was used to show nuclear staining. Localization of the different constructs was observed by fluorescence microscopy. Graphs with the percentage of cells with the different localization are shown on the right. N, Nuclear localization, C, Cytoplasmic localization, N=C, Nuclear and cytoplasmic distribution within the same cell is equal, N>C, Nuclear localization is more intense than cytoplasmic localization, N<C, Nuclear localization is less intense than cytoplasmic localization, and PN, perinuclear localization.  
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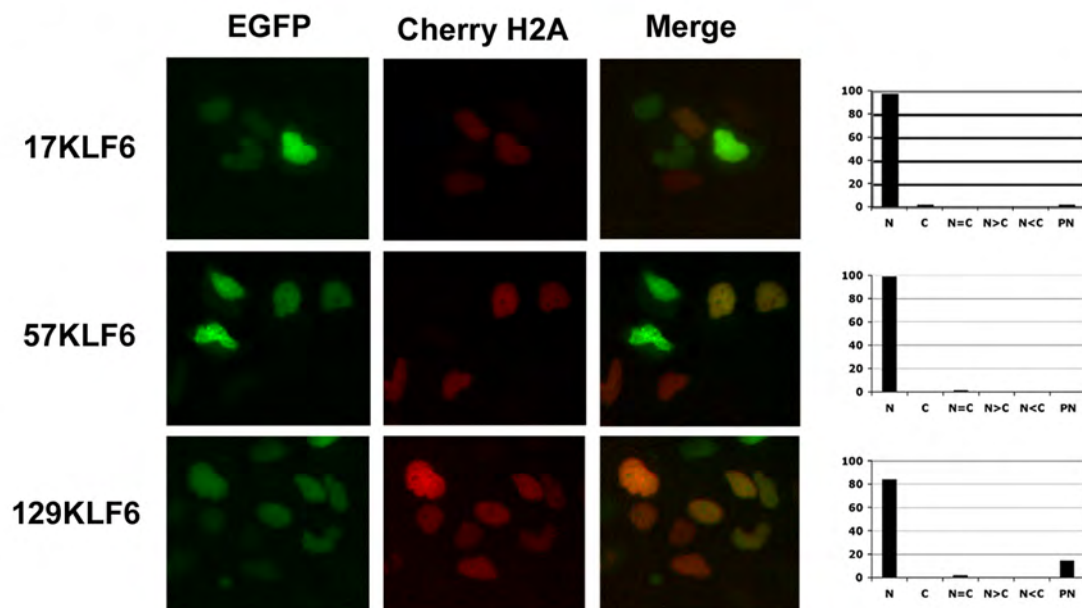
study we demonstrated that mutations in amino acids M6 and R243 result in either nuclear localization or cytoplasmic sequestration of KLF6, respectively (Figure 3B, Figure S2 and Figure 8). Given that we demonstrated M6A mutant to have increased nuclear localization, it will be of interest to specifically functionally interrogate the patient-derived KLF6 and KLF6-SV1 M6V mutants, which would both share the mutation, to better

understand if their association with cancer arises from loss of the tumor suppressor or activation of the oncogenic variant.

Beyond mutational inactivation, dysregulation of KLF6 alternative splicing has also been described in a number of cancers and increased production of KLF6-SV1 is associated with increased tumor stage [3], chemoresistance [51] and poor prognosis [52]. In one sense, KLF6-SV1 represents a naturally occurring inactivating



**B**

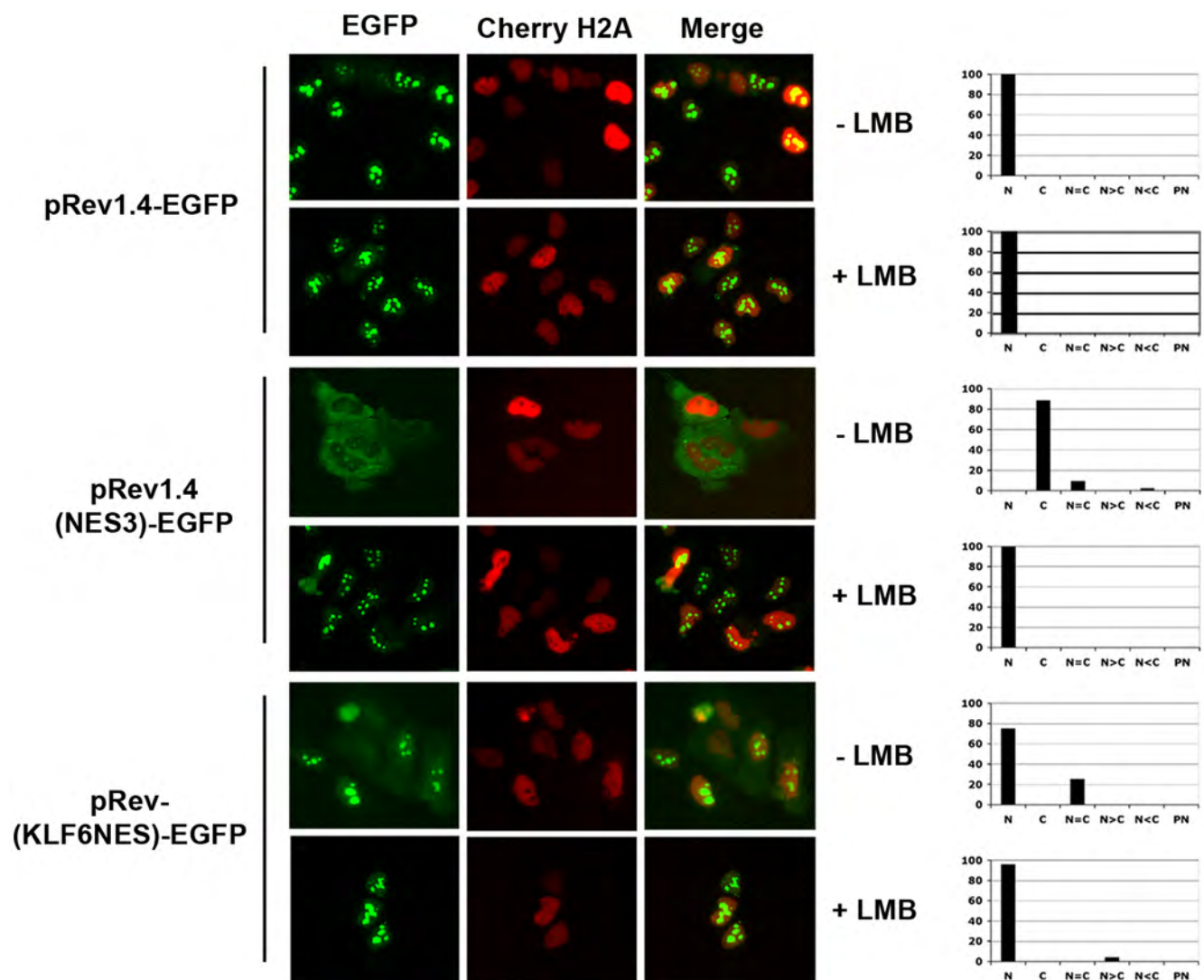


**Figure 4. Identification of a Crm1-dependent KLF6/KLF6-SV1 nuclear export signal.** A, HeLa cells transfected with EGFP-KLF6, EGFP-KLF6-SV1 or empty vector were treated with or without LMB for 2 h. B, The subcellular localization of truncated KLF6 constructs is shown. Cherry-H2A construct was used to show nuclear staining. Localization of the different constructs was observed by fluorescence microscopy. Graphs with the percentage of cells with the different localization are shown on the right. N, Nuclear localization, C, Cytoplasmic localization, N=C, Nuclear and cytoplasmic distribution within the same cell is equal, N>C, Nuclear localization is more intense than cytoplasmic localization, N<C, Nuclear localization is less intense than cytoplasmic localization, and PN, perinuclear localization.  
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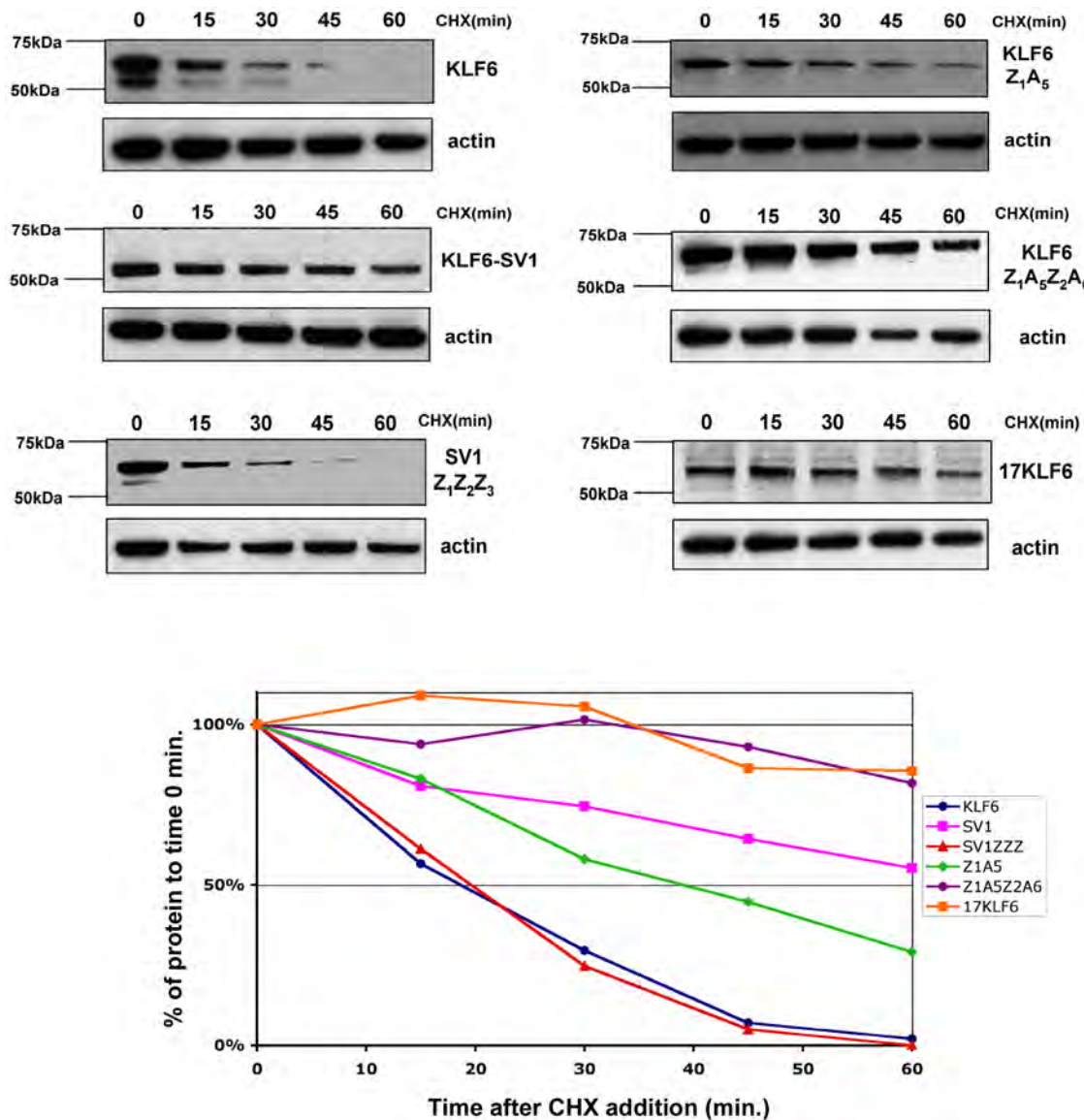
mutation of the KLF6 NLS. Thus the antagonistic functions of these two proteins can in part be related to their distinct subcellular localizations.

Our demonstration that the KLF6 zinc fingers also encode the functional NLS provides further support for the hypothesis by Pandya *et al.* [21] that a common NLS is present in the zinc finger domain of all KLF family members. Distinctions between the domains do however exist. Different from KLF1 and KLF4 where all three ZFs appear to be necessary for nuclear localization as well

as the 5' basic region adjacent to them [20–22], ZF<sub>1</sub> plays the main role in defining KLF6 subcellular localization (Figure 2). Similar results demonstrating a functional role for ZF<sub>1</sub> have also been recently demonstrated for KLF8 [23]. On the other hand, ZF<sub>2</sub>, ZF<sub>3</sub> and the KLF6 5' basic region sequence (PDGRRRVHR) are not sufficient to direct nuclear localization (Figure 2). These results in deconstructing the functional roles of each zinc finger are in accord with and help to explain previously published findings on the subcellular localization of other KLF6



**Figure 5. KLF6 presents a CRM1-dependent NES that is of relatively weak strength.** EGFP localization in HeLa cells co-transfected with Cherry-H2A and wild type Rev protein (pRev1.4-EGFP), a NLS mutant Rev protein (pRev1.4-(NES3)-EGFP) or a Rev carrying KLF6 NES (pRev-(KLF6NES)-EGFP). Cells were treated or not with LMB for 2 h. Both EGFP and the corresponding fields for Cherry-H2A are shown. Graphs with the percentage of cells with the different localization are shown on the right. N, Nuclear localization, C, Cytoplasmic localization, N=C, Nuclear and cytoplasmic distribution within the same cell is equal, N>C, Nuclear localization is more intense than cytoplasmic localization, N<C, Nuclear localization is less intense than cytoplasmic localization, and PN, perinuclear localization.  
doi:10.1371/journal.pone.0012639.g005



**Figure 6. Effects of KLF6 nucleo-cytoplasmic localization domains on protein half-life.** Western-blots showing half-life experiments for the wild type and different NLS and NES mutants. Cells were harvested at the times indicated after CHX treatment. Membranes were probed with anti-GFP to detect KLF6, KLF6-SV1 and the mutants, and with anti-actin as a loading control. The graph represents the values obtained after densitometry analysis. The percentage of remaining protein after CHX addition is plotted.  
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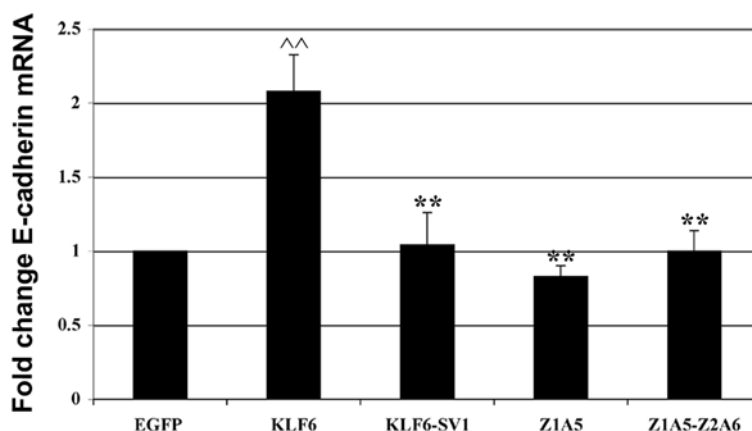
splice variants. Splice variant 2 (KLF6-SV2), which lacks ZF1 but possesses ZF2 and ZF3, localizes in the cytoplasm [16]. Splice variant 3 (KLF6-SV3), which maintains ZF1 but not ZF2 and ZF3 localizes to the nucleus (Martignetti and Camacho-Vanegas, unpublished results).

Recently, Du *et al.* [24] described the presence of an NES in a KLF family member. The KLF5 NES was shown to be Crm1-dependent and present between aa 119–139 within the regulatory domain and located near a SUMO motif that regulates nuclear export. In this work, we describe that the first 16 amino acids of the common KLF6 and KLF6-SV1 protein sequence contain a NES that might be Crm1-dependent because KLF6 is entrapped in the nucleus following treatment with LMB. Targeted deletions and mutations in some of the hydrophobic residues within this 16 aa domain also resulted in increase in nuclear accumulation. In comparing the strength of the NES to the well-characterized Rev

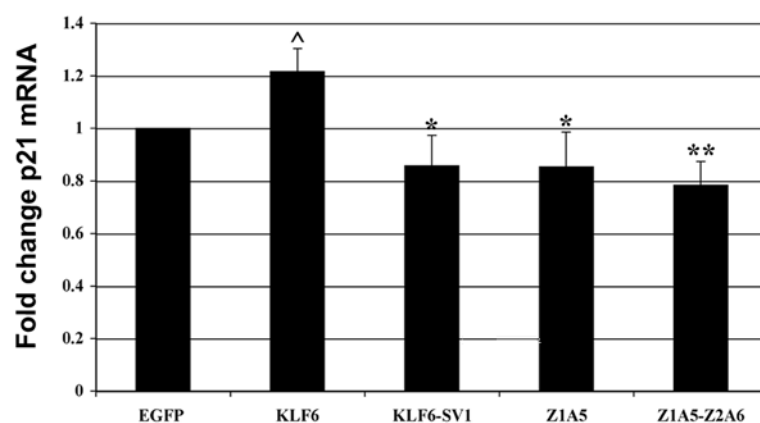
protein, the KLF6 NES was shown to be weaker and thus similar to that of other transcription factors such as p53 and p53-regulated genes like p21 and Hmd2 [45].

One unexpected finding from these studies was the observation that KLF6-SV1, which lacks the KLF6 NLS and which we have previously shown to be localized primarily in the cytoplasm [16] was nonetheless found to be partially relocalized to the nucleus when cells were treated with LMB, a Crm1 inhibitor. This suggests that KLF6-SV1 can be transported into the nucleus in an NLS-independent manner, possibly through binding KLF6 or other actively nucleo-cytoplasmic shuttled proteins (*piggy-backing*). This has been shown to occur with other tumor suppressors including BRCA1, whose NLS-lacking alternatively splice isoforms are transported into the nucleus following DNA damage [53]. In this instance, nuclear transport is mediated through binding to BARD1, another tumor suppressor that heterodi-

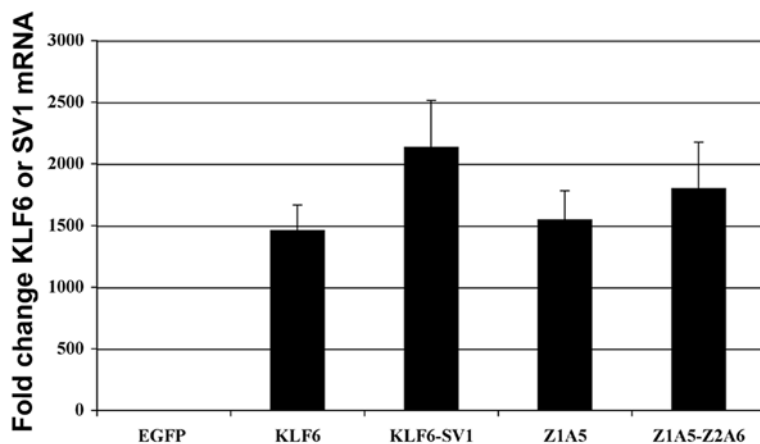
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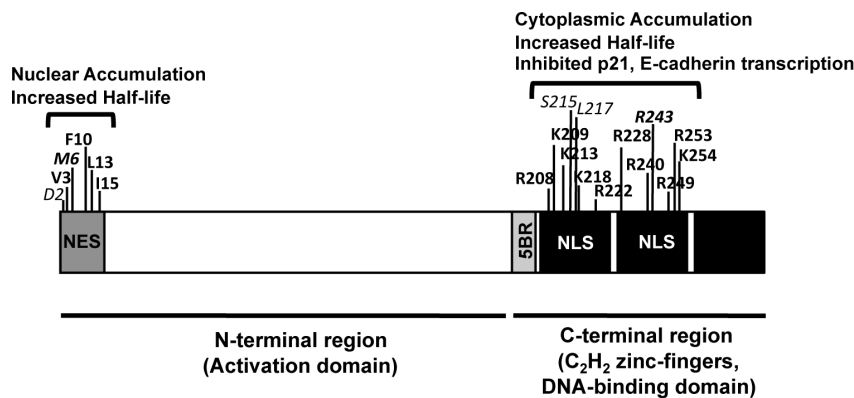
**Figure 7. KLF6 intact NLS is necessary for KLF6 tumor suppressor function.** RT-PCR data showing endogenous levels of E-cadherin (panel A), p21 (panel B) and the different constructs (panel C). Expression levels were calculated by normalizing each cDNA to GAPDH and then using this normalized value to calculate fold change to the EGFP empty vector value. All experiments were performed at least three times and in triplicate. Statistical significance was determined by two tailed, two-sample equal variance T-test (^=p<0.05 and ^^=p<0.005 to EGFP; \*=p<0.05 and \*\*=p<0.005 to EGFP-KLF6).

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merizes with BRCA1 to form a complex involved in DNA damage repair [53].

Our results link, for the first time, nucleo-cytoplasmic transport of a KLF family member to protein stability. Given KLF6's tumor suppressor function and KLF6-SV1's oncogenic/anti-apoptotic function, this finding may have broad implications. Previous

studies showed that KLF6 is ubiquitinated and degraded via the proteasome and has a short half-life of ~15 min [47]. KLF6-SV1 half-life is appreciably longer [54]. The mechanisms underlying their turnover remained unknown. Here we demonstrate that regulated turnover requires an intact NLS and NES. Disruption of either of them modified KLF6 protein stability. Furthermore,



**Figure 8. Site-directed and patient-derived mutations in the NES and NLS and their consequences.** Site-directed mutations are highlighted in bold, whereas patient-derived mutations, described in the text, are italicized. Overlapping mutations are shown in both bold and italics. For previously published data check reference (55). doi:10.1371/journal.pone.0012639.g008

addition of the NLS to KLF6-SV1 not only restored nuclear localization but also decreased protein stability, resulting in a protein with a half-life more similar to wild-type KLF6.

In further agreement with our hypothesis that regulation of nucleo-cytoplasmic transport is a critical determinant of KLF6 function, we demonstrated that mutations in the KLF6 NLS domain result in decreased transcriptional activation of two cancer-relevant targets, p21 and E-cadherin. Access to the nuclear compartment might be a first step of regulation prior to activating target promoters. This has also been demonstrated recently for another KLF member, KLF8, in which the presence of an intact NLS is needed for increased Cyclin D1 transcriptional activation and increased cell proliferation [23]. Ultimately, and given the demonstrated role of KLF6 and KLF6-SV1 in human cancers, it will be important to examine the possible post-translational modifications which may provide additional layers of regulation to their nucleo-cytoplasmic regulation as well as the mechanism(s) which allow NES-independent KLF6-SV1 nuclear import. The regulation and cellular consequences of nuclear KLF6-SV1 remain to be determined.

## Materials and Methods

### Generation of plasmids and site-directed mutagenesis constructs

The pEGFP-KLF6 plasmid was generated by amplifying the complete KLF6 coding sequence from the pCIneo-KLF6 construct [1] using the primers fwd-KLF6pCIneo and rev-KLF6pCIneo (Table S1). The resulting amplicon was then subcloned using EcoRI sites into the pEGFP-C3 vector (Clontech). The pEGFP-KLF6-SV1 plasmid was generated by cloning the entire KLF6-SV1 coding sequence obtained by EcoRI enzymatic restriction digest from the pCIneo-KLF6-SV1 vector [48] into pEGFP-C3. The pEGFP-5BR construct contains the KLF6 putative NLS sequence (PDGRRRVHR) that was cloned EcoRI/BamHI in pEGFP-C3 from annealing of complementary forward and reverse primers (Table S1). The pEGFP-ZF<sub>1</sub>ZF<sub>2</sub>ZF<sub>3</sub> construct was made using the primers fwd-Z<sub>1</sub>Z<sub>2</sub>Z<sub>3</sub> and rev-Z<sub>1</sub>Z<sub>2</sub>Z<sub>3</sub> (Table S1) to amplify KLF6 zinc fingers (ZF) from the pCIneo-KLF6 vector and then cloned BamHI into the pEGFP-C3 vector. Plasmids pEGFP-ZF<sub>1</sub>, pEGFP-ZF<sub>2</sub> and pEGFP-ZF<sub>3</sub>, carrying individual KLF6 ZFs, were obtained by cloning KLF6 ZF<sub>1</sub> (BamHI), ZF<sub>2</sub> (EcoRI/BamHI), and ZF<sub>3</sub> (EcoRI/BamHI) sequences amplified by PCR from the pCIneo-KLF6 construct and

using the primers fwd-Z<sub>1</sub>Z<sub>2</sub>Z<sub>3</sub>/rev-Z<sub>1</sub>, fwd-Z<sub>2</sub>/rev-Z<sub>2</sub> and fwd-Z<sub>3</sub>/rev-Z<sub>1</sub>Z<sub>2</sub>Z<sub>3</sub> (Table S1), respectively. The pEGFP-SV1-Z<sub>1</sub>Z<sub>2</sub>Z<sub>3</sub> construct was obtained by cloning KLF6-SV1 coding sequence into the pEGFP-ZF<sub>1</sub>ZF<sub>2</sub>ZF<sub>3</sub> plasmid digested with EcoRI.

We generated the N-terminus deletion constructs lacking the first 128 (pEGFP-129KLF6), 56 (pEGFP-57KLF6) and 16 (pEGFP-17KLF6) amino acids (aa), using the primer combinations fwd-129-283/rev-KLF6pCIneo, fwd-57-283/rev-KLF6pCIneo and fwd-17KLF6/rev-KLF6pCIneo, respectively (Table S1). EcoRI digested amplicons were then subcloned into the pEGFP-C3 vector.

Point mutations in the NES and NLS were sequentially generated in the pEGFP-KLF6 plasmid using commercially available kits following the manufacturer's recommendations (Stratagene, USB Corporation) and the primers listed in Table S2.

The construct pRev-(KLF6NES)-EGFP was made by cloning annealed primers containing the KLF6 NES sequence in the plasmid pRev1.4(NES3)-EGFP digested with BamHI/AgeI. Plasmids pRev1.4 (NES3)-EGFP and pRev1.4-EGFP were kindly donated by Dr. Eric Henderson (Westmead Institute for Cancer Research, Sydney, Australia).

All primer sequences are shown in Table S1 and S2. All expression constructs were confirmed by DNA sequencing in both orientations prior to their use.

### Growth and maintenance of cell lines

All cell lines were purchased from the American Type Culture Collection (Manassas, VA). Cells were grown and maintained in DMEM media (Cellgro<sup>®</sup>) supplemented with 10% FBS (Gibco) and 1% Penicillin/Streptomycin (Cellgro<sup>®</sup>). Cells were transfected with Lipofectamine<sup>™</sup> 2000 reagent according to the manufacturer's recommendations (Invitrogen).

### Western blot and half-life analysis

Protein extracts for Western blotting were obtained by lysing the cells with radioimmunoprecipitation assay buffer following standard protocols. Protein concentration was measured using the Bio-Rad DC Protein quantification assay and amounts adjusted such that equivalent amounts were loaded (7.5 ug). Electrophoresed proteins were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. Blots were blocked with 5% non-fat milk (LabScientific, Inc.) in TBS-Tween buffer. We used the following primary antibodies: Actin (I-19) (Santa Cruz Biotechnology) and GFP Living Colors (JL-8) (Clontech). Both primary and secondary

antibodies were incubated at a dilution of 1:1000 in 5% non-fat milk in TBS-Tween.

For the half-life experiments, HeLa cells were transfected with different constructs. The next day, transfected cells were treated with 1 mg/ml of Cycloheximide (Sigma). Protein extracts were obtained at the noted times and then analyzed by Western-blot.

### Fluorescent microscopy

EGFP subcellular localization was observed using a fluorescent microscope (NIKON Eclipse TE 200) with a 20× objective. Photomicrographs were acquired using Spot Advanced Software and the Image J program.

For all localization experiments, two wells of a 6-well plate were transfected and analyzed for each EGFP construct. At the minimum, six [6] fields were randomly chosen and green cells were counted in order to calculate the percentages of nuclear (N), cytoplasmic (C) and perinuclear (PN) cells. In addition, each experiment was repeated at least three times.

### RNA extraction and quantitative real time-PCR (qRT-PCR) analysis

RNA extraction and qRT-PCR analysis were done as previously described [51]. Briefly, RNA was obtained from cells using the Rneasy Mini kit (Qiagen) and treated with DNase (Qiagen). One ug of RNA was used in each reaction to obtain the first-strand complementary DNA by reverse transcription using random primers (Promega). An ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) was used for the qRT-PCR. The primer sequences used have been previously described [3,16,48]. All values were normalized to GAPDH levels. All experiments were performed in triplicate and validated thrice independently. Statistical significance was determined by two tailed, two-sample equal variance *T*-test ( $\wedge = p < 0.05$  and  $\wedge\wedge = p < 0.005$  to EGFP;  $\ast = p < 0.05$  and  $\ast\ast = p < 0.005$  to EGFP-KLF6).

### Luciferase transactivation assays

HeLa cells transfected with a p21 promoter construct (1 ug) and either KLF6, KLF6-SV1, the NLS mutants or EGFP empty vector (1 ug) were harvested 24 h after transfection. Dual-Luciferase® Reporter Assay kit (Promega, Madison, WI, USA) was used to extract protein and develop the assay following the manufacturer's recommendations. The TK promoter-Renilla Luciferase construct (Promega, Madison, WI, USA), 10 ng, was used to normalize each experiment. Luciferase activity was determined for each EGFP construct by luminescence in a Modulus™ II Microplate Multimode Reader (Promega, Madison, WI, USA). All experiments were performed in triplicate and validated thrice independently. Statistical significance was determined by two tailed, two-sample equal variance *T*-test ( $p < 0.005$ ).

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### Supporting Information

**Figure S1** Localization of KLF6, KLF6-SV1, KLF6 NLS and the ZFs in 293T cells. Co-localization of KLF6, KLF6-SV1, KLF6 NLS or the ZFs EGFP constructs together with Cherry-H2A, which was used to show nuclear staining. Localization of the different constructs was observed by fluorescence microscopy. Found at: doi:10.1371/journal.pone.0012639.s001 (1.17 MB TIF)

**Figure S2** Mutations in the N-terminus 16 amino acids results in increased KLF6 nuclear localization. Subcellular localization of the different NES mutants. Cherry-H2A construct was used to show nuclear staining. Localization of the different constructs was observed by fluorescence microscopy. Graphs with the percentage of cells with the different localization are shown on the right. N, Nuclear localization, C, Cytoplasmic localization, N = C, Nuclear and cytoplasmic distribution within the same cell is equal, N > C, Nuclear localization is more intense than cytoplasmic localization, N < C, Nuclear localization is less intense than cytoplasmic localization, and PN, perinuclear localization. Found at: doi:10.1371/journal.pone.0012639.s002 (1.62 MB TIF)

**Figure S3** p21 promoter luciferase assays for KLF6, KLF6-SV1 and the NLS mutants as well as EGFP empty vector in 293T cells. Expression levels were calculated by normalizing each luciferase value to Renilla gene expression and representing the Relative Luciferase Units (RLU). All experiments were performed at least three times and in triplicate. Statistical significance was determined by two tailed, two-sample equal variance *T*-test ( $p < 0.005$ ). Found at: doi:10.1371/journal.pone.0012639.s003 (0.08 MB TIF)

**Table S1** Primers used for site-directed mutagenesis. 'P' represents the primers that are 5' phosphorylated. Found at: doi:10.1371/journal.pone.0012639.s004 (0.04 MB DOC)

**Table S2** Primers used to generate expression constructs. Restriction sites are underlined. Found at: doi:10.1371/journal.pone.0012639.s005 (0.04 MB DOC)

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### Author Contributions

Conceived and designed the experiments: ER JAM. Performed the experiments: ER NA NMP. Analyzed the data: ER NA NMP AD JAM. Contributed reagents/materials/analysis tools: JAM. Wrote the paper: ER JAM.

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# The Krüppel traffic report: Cooperative signals direct KLF8 nuclear transport

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The Krüppel-like transcription factor (KLF) family consists of 17 distinct family members involved in the regulation of diverse cellular processes including differentiation, cell proliferation, growth-related signal transduction, angiogenesis and apoptosis (recently reviewed in [1]). In addition to their currently known biologic roles, the discovery that at least one member of the family, KLF6, can be alternatively spliced into biologically active isoforms with antagonistic functions and a distinct subcellular localization pattern [2], highlights the fact that nuclear-cytoplasmic shuttling of KLF proteins may represent an additional layer of functional regulation and the possibility of an even more diverse and completely unexplored role for KLF family members in both health and disease.

The study by Mehta *et al.* [3] on KLF8 nuclear localization provides additional and novel findings on the signals and cooperativity between them which direct subcellular trafficking of KLF family members. Prior to discussing these findings it is worthwhile to review the basic modular structure that has been “classically” described for this family. Based on sequence comparison, the modular structure is shown to be retained even in evolutionarily distant

homologues including those in *Zebrafish* [4], *Xenopus* [5] and *Drosophila* [6]. A central feature ascribed to all KLF family members, if alternative splicing is not considered, has been their possession of three characteristic domains. The first is a highly variable N-terminal activation domain. Post-translational modifications within this domain and interactions with other proteins through this domain are believed to underlie each KLF family member’s ability to act as either an activator or repressor of transcription. Second, a C-terminal region containing three highly conserved C<sub>2</sub>H<sub>2</sub> zinc fingers (ZFs) comprises the DNA binding domain. Finally, based originally on the presence of an enriched stretch of basic amino acids, a nuclear localization signal (NLS) region was predicted adjacent to the start of the zinc finger DNA-binding domain (Figure 1).

Functional NLSs were first demonstrated in KLF1 and KLF4. Surprisingly, while the NLS sequence was shown to be functional in each of these proteins [7, 8], the ZFs also were involved in determining subcellular localization. Specifically, all KLF1 ZFs were found to be necessary and sufficient to localize KLF1 in the nucleus as shown by either deletion [9] or different fusions of the ZFs to GFP [8]. For KLF4, all three ZFs together were also enough to localize GFP to the nucleus. In addition, deletion constructs revealed that combined

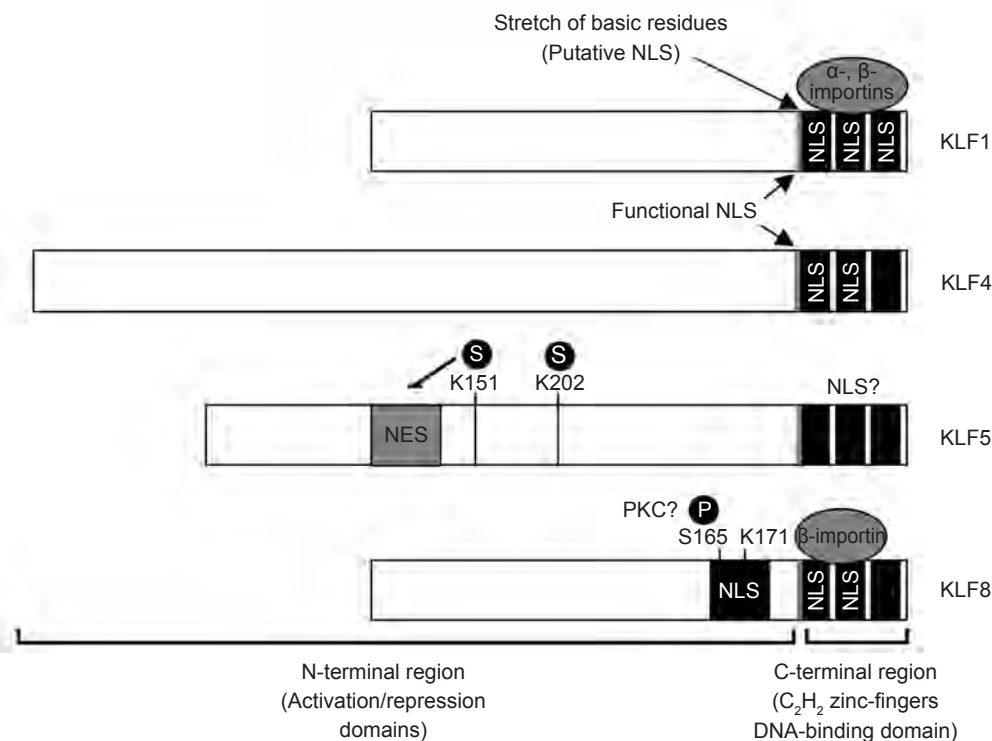
ZF1 and the first part of ZF2 were also sufficient to localize GFP to the nucleus. When tested individually, each ZF could direct nuclear localization, however ZF3 alone was the weakest and its deletion from the full-length protein had no effect on nuclear localization [7].

More recent studies demonstrated that SUMOylation of KLF5 increases nuclear localization by inhibiting nuclear export signal (NES) activity. Mutations of the residues K151 and K202, which are located near an NES, inhibit SUMOylation, resulting in mislocalization of KLF5 to the cytoplasm. Moreover, this post-translational modification also influences protein activity. The mislocalized mutants, unlike the wild type protein, lose the ability to promote anchorage-independent growth [10].

The authors from Mehta *et al.* [3] previously reported that KLF8 is also SUMOylated [11]. Different than KLF5, SUMOylation does not affect KLF8 nuclear localization but does regulate its function as a transcriptional repressor. To date, no other studies have addressed the regulation of KLF8 subcellular localization. Therefore, the study by Mehta *et al.* now provides some novel insights into the regulation of this transcription factor’s nucleocytoplasmic transport.

Previous studies based on sequence homology have described two putative NLSs in KLF8 [12]. One of them is located immediately upstream of the

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**Figure 1** Modular structure of the KLF family members and domains controlling their subcellular localization. The cartoon shows the functional domains and post-traslational modifications related to nucleo-cytoplasmic transport identified in KLF1, KLF4, KLF5 and KLF8 [3, 7, 8, 9, 10]. NLS stands for Nuclear Localization Signal and NES, Nuclear Export Signal. The circles with an S indicate those residues that are SUMOylated, and the circle with a P, that the residue is phosphorylated. Binding to  $\alpha$ - and  $\beta$ -importins is also shown.

ZFs (mNLS1), which corresponds to the stretch of basic amino acids present in other KLFs. The other, mNLS2, is located at the carboxy terminal of the KLF8 protein sequence. Mehta *et al.* demonstrated that these two sequences do not play a role in KLF8 nuclear transport as either deletion or mutation does not change KLF8 nuclear localization.

Instead, through a series of experiments Mehta *et al.* defined the presence of two functional and cooperative NLSs in KLF8 distinct from the originally presumed motifs [3]. The first, similar to what has been previously described for KLF1 and KLF4, was located within the ZF domain. Unlike the NLS in KLF1, in which all ZFs contribute to KLF1 nuclear localization, only the first two ZFs are required for nuclear transport. Deletion of either or both ZF1 and

ZF2 in the full-length protein increases cytoplasmic localization. Similar to KLF4, targeted loss of ZF3 has no effect on subcellular distribution whereas similar to KLF1, KLF8 binds  $\beta$ -importin through interaction with its ZFs [8].

The second functional KLF8 NLS is novel and unique to this transcription factor. It is located within the N-terminal activation domain, between amino acids (aa) 151-200. The authors demonstrated that deletion of this region results in cytoplasmic mislocalization. Moreover, this region contains two residues, S165 and K171, which seem to play a role in the regulation of KLF8 nuclear transport, as mutations of either increased KLF8 cytoplasmic localization. The S165 residue was suspected to be a PKC modification domain based on sequence similarity and indeed, treat-

ment with a PKC inhibitor decreased nuclear localization. Future experiments will be necessary to elucidate whether PKC directly phosphorylates KLF8 and whether other post-traslational modifications may also control nucleo-cytoplasmic shuttling.

What functional consequences, if any, result from changes in KLF8 localization? As would be expected for a transcription factor, subcellular localization should be a critical determinant of function. KLF8 has been suggested to play important roles in human tumorigenesis through its ability to induce both cell cycle progression via activation of cyclin D1 [12] and in promoting epithelial to mesenchymal transition, oncogenic transformation and invasion [13, 14]. In part, this is regulated by transcriptional induction of KLF8

expression through activation of the focal adhesion kinase (FAK) pathway [12]. Therefore, dysregulation – for example, through mutation in tumor cells – may be an important, although as of yet unreported, pathogenic mechanism. In accord with this, Mehta *et al.* demonstrated that KLF8 requires both NLSs to up-regulate cyclin D1 expression and that mutations in S165 and K171 result in significant decreases in cyclin D1 promoter induction. These results correlate well with the results obtained by BrdU incorporation assays, which demonstrated decreased cellular proliferation compared to the wild type protein.

In summary, the study by Mehta *et al.* is the first to demonstrate that cooperation between both functional NLSs is necessary to regulate KLF8 nuclear transport and that nuclear localization is necessary for correct functioning as a transcription factor [3]. Therefore, despite the high degree of homology that all KLF proteins share within their ZF domains, it is becoming clear that their contribution to nuclear localization cannot simply be considered equal: the nuclear “traffic signals” encoded within these domains, while highly similar on a sequence level, are not functionally identical. Thus, beyond their current sequence-based [15], to truly appreciate the biology of this family, additional biochemical studies are needed to understand the rules of the road which

ultimately regulate nucleo-cytoplasmic traffic of these transcription factors.

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